STUDIES ON IMMUNITY IN ANTHRAX

X. Gel-Adsorbed Protective Antigen for Immunization of Man

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ABSTRACT

PUZISS, MILTON (Fort Detrick, Frederick, Md.) and GEORGE G. WRIGHT. Studies on immunity in anthrax. X. Gel-adsorbed protective antigen for immunization of man. J. Bacteriol. **85**:230–236. 1963.—Protective antigen in filtrates of anaerobic cultures of *Bacillus anthracis* was not readily precipitable by alum or other aluminum salts, but was adsorbed onto a preformed aluminum hydroxide gel under specific conditions. The adsorbed product was highly effective in immunizing rabbits, guinea pigs, and mice against challenge with virulent *B. anthracis* spores. Gel-adsorbed anaerobic antigens preserved with 1:10,000 thimerosal were unstable on storage at 4 C; replacement of this preservative with 1:40,000 benzethonium chloride produced a more stable product. Addition of 0.009% formalin further increased the stability during accelerated aging at 37 C. The final product had high protective activity in animals and was well tolerated in man.

The protective antigen of *Bacillus anthracis*, produced under aerobic conditions and precipitated by alum, was shown to immunize a variety of animals against lethal challenge with *B. anthracis* (Wright, Green, and Kanode, 1954). The alum-precipitated antigen significantly reduced the incidence of anthrax in an occupationally exposed group of volunteers (Brachman et al., 1962). Subsequently, elaboration of antigen was demonstrated under anaerobic conditions, and further modifications of the medium and use of more productive strains resulted in production of culture filtrates of significantly higher protective antigenicity than had been obtained previously (Wright, Puziss, and Neely, 1962). This paper describes studies on adsorption of antigen from filtrates of anaerobic cultures, and the development of a stable preparation of antigen that had high protective activity in animals and was well tolerated in man.

MATERIALS AND METHODS

Cultures. The nonencapsulated, nonproteolytic strains of *B. anthracis* used for antigen production and the method of preparing and carrying stock strains were described previously (Wright et al., 1962). A standard spore suspension of the virulent Vollum strain of *B. anthracis* was used for challenge of immunized animals.

Medium and methods of anaerobic culture. The basal medium used for anaerobic growth was the 1095 medium of Wright et al. (1962). This medium was prepared in aspirator bottles in volumes ranging from 4 to 10 liters. After the medium was inoculated, the cotton plug on the culture vessel was replaced by a sterile rubber stopper fitted with two cotton-packed air filters. This assembly was securely fastened to the culture vessel, because of the slight internal pressure that developed during incubation. Nitrogen was introduced through one of the filters for 10 min to displace air, and the vessel was sealed off with screw clamps, thereby retaining an atmosphere of nitrogen above the medium during incubation. After 18 hr of incubation at 37 C, the magnetic stirring motor was turned on, and incubation continued until the culture was harvested at 42 hr, unless otherwise noted.

Preparation and assay of protective antigen. The pH of the culture, which had dropped to 6.8 to 7.0 from an initial level of 7.8, was adjusted to 8.0 with 1 N NaOH prior to filtration. For filtration of 10-liter volumes of culture, four 4-liter vacuum flasks were each fitted by means of rubber stoppers with large (1.75 × 13.75 in.) ultrafine sintered-glass candles (Corning) encased in glass mantles. The mantles were connected by a glass manifold to the tubulature of the culture vessel. Culture flowed by gravity into the mantles, and vacuum was applied to the receiver

flasks; by this means, 10 liters of culture filtered in approximately 60 min without prior centrifugation.

Filtrates were tested for sterility, and were either used immediately for precipitation studies or were lyophilized and stored in a desiccator. The yield on lyophilization ranged from 4.5 to 5 mg per ml of culture filtrate. The lyophilized filtrates were reconstituted as required to provide reproducible material for precipitation studies. Protective antigen in culture filtrates was measured by immunization and challenge of guinea pigs (Puziss and Wright, 1959), by complement-fixation titrations (McGann, Stearman, and Wright, 1961), and by the Ouchterlony double-diffusion method (Thorne and Belton, 1957). Precipitated or adsorbed antigen was assayed in 3- to 5-lb black Dutch rabbits, obtained from a closed colony at Fort Detrick. The animals were immunized subcutaneously with a single 0.5-ml injection of antigen, diluted with 0.9% sodium chloride solution (saline) as indicated. Immunized rabbits and normal control animals were challenged 14 days later with approximately 10,000 spores of the virulent Vollum strain of *B. anthracis*, given intracutaneously in a volume of 0.25 ml. This challenge dose represented between 500 and 1,000 LD_{50}, and was invariably fatal to normal rabbits.

Adjuvants. Aluminum phosphate suspension, the "7/8 gel" of Holt, was prepared as described by Wright et al. (1960). The authors are indebted to H. B. Devlin, of Parke, Davis & Co., Detroit, Mich., for this material. Three preparations of aluminum hydroxide gel were used. Amphojel, produced by Wyeth Laboratories, Inc., Radnor, Pa., contained colloidal aluminum hydroxide equivalent to 4% Al_2O_3. Gel developed specifically for use in human biologicals was prepared according to the method of Hansen (1953). The authors are indebted to Inga Scheibel of the Statens Seruminstitut, Copenhagen, Denmark, for one lot of this material, designated lot N; a similar preparation produced in our laboratory is designated lot FD.

Preservatives. Benzethonium chloride USP (Phemerol, produced by Parke, Davis & Co.) was initially purchased as a 1:1,000 solution. For later work, and for all preparations of antigen used in human immunization, the crystalline compound was provided by Parke, Davis & Co., through the courtesy of H. B. Devlin. Methyl and propyl parabenzoic acids were obtained from the Heyden Chemical Co., New York, N.Y.

RESULTS

Precipitation of antigen. Attempts were made to concentrate and stabilize the protective antigen in anaerobic culture filtrates by precipitation with alum according to the procedure that had been successful with aerobic filtrates (Wright et al., 1954). This method of precipitating antigen was not satisfactory with anaerobic filtrates; despite the high antigen content of the filtrate, the antigenicity of the precipitated product was not as high as that derived from aerobic cultures. Extensive variation of such factors as concentration of alum and pH of precipitation, prior dialysis of culture filtrate, or addition of 0.04% protamine sulfate were all unsuccessful in obtaining alum-precipitated preparations of uniformly high protective activity. Unsatisfactory results were likewise obtained with aluminum chloride, which was added to reconstituted culture filtrate at a final concentration of 0.17% and adjusted to pH values ranging from 5.95 to 8.0. None of the resuspended precipitates showed significant protective activity in rabbits. Adsorption of antigen onto preformed "7/8" aluminum phosphate gel of Holt (Wright et al., 1960) was studied at pH values ranging from 6.0 to 8.0. Occasional preparations showed significant protective activity in rabbits, but the results were irregular.

Adsorption of antigen by aluminum hydroxide. Exploratory experiments revealed that aluminum hydroxide gel (Amphojel, Wyeth Laboratories, Inc.) had considerable adsorptive capacity for protective antigen, and more detailed investigations were undertaken to determine the effect of different conditions of adsorption. It was found that optimal adsorption of antigen with this gel took place at pH 8.0, rather than at pH 5.9, which had produced optimal results with alum precipitation. The culture had been adjusted to pH 8 prior to filtration, so no further adjustment of the filtrate was necessary. The lowest concentration of gel that yielded adsorbed products of essentially maximal antigenicity was 0.041%, calculated as Al_2O_3. The suitably diluted stock gel was autoclaved and added to the culture filtrate at a concentration of 25 ml per liter. A large flocculent precipitate, which settled at a moderately rapid rate, was formed immediately. The gel-adsorbed antigen was allowed to stand for 7 days at 4 C,

TABLE 1. *Elaboration of antigen by several strains of Bacillus anthracis*

Strain	Complement fixation titer of culture filtrate	Immunizing activity in rabbits*
	50% units/ml	
107-NP2-R1	160	11/12
V770-NP1-R	160	24/28
116-NP1-R	140	3/4
Weybridge	160	4/5
Unimmunized controls		0/8

* Gel-adsorbed antigen preparations were diluted 1:10 with saline for immunization of animals. Immunizing activity is expressed as the number of animals surviving divided by the total number challenged.

with agitation for several minutes each day. The supernatant fluid was then drawn off and discarded, the gel was packed by low-speed centrifugation (750 × *g* for 15 min), and the remaining supernatant was discarded. The gel-adsorbed antigen was resuspended in cold physiological saline to 0.1 of the volume of the original filtrate. Thimerosal (1:10,000) was added as a preservative. The two aluminum hydroxide gels prepared for use in biologicals by the method of Hansen (1953), lot N and lot FD, proved to be equivalent to the commercially available gel in antigen-adsorbing ability; settling characteristics were the same. The lowest satisfactory concentration of these gels was 0.031%.

An experiment was performed to determine the effect of agitation on adsorption of antigen on the gel. The adsorption was allowed to proceed at 4 C, either with resuspension by shaking once each day, or with continuous slow agitation by means of a magnetic stirrer. Samples were taken at intervals, centrifuged, and the precipitates resuspended in saline as before. Tests for antigenicity in rabbits revealed that continuous stirring accelerated adsorption of antigen, so that adsorbed products of high antigenicity were obtained after 2 days. This procedure was used for adsorption of antigen in subsequent work. Culture filtrates derived from several strains were adsorbed according to this method, and preparations with high protective activity in rabbits were obtained in each case (Table 1).

Stability of the gel-adsorbed antigen. Reassay of a number of initially active antigen preparations

TABLE 2. *Loss of antigenicity of gel-adsorbed protective antigen during storage at 4 C*

Antigen prepn	Storage period at 4 C	Immunizing activity*
	months	
2360-5	0	4/4
	2	3/4
	8	0/4
2372-6	0	4/4
	1	4/4
	6	0/4
2372-7	0	3/4
	1	3/4
	6	0/4
2402-1	0	3/4
	2	0/4

* Antigen for immunization was diluted 1:10 with saline. Immunizing activity is expressed as the number of rabbits surviving divided by the total number challenged.

revealed that the antigenicity fell off rapidly during storage at 4 C (Table 2). This was in marked contrast to the stability of aerobic culture alum-precipitated antigens, some of which had maintained their antigenicity for periods of 3 years or more at 4 C. Lyophilization of gel-adsorbed or alum-precipitated antigen was unsuccessful as a method for increasing stability, since the reconstituted material was difficult to resuspend and its antigenicity was very low.

In an effort to understand the mechanism of loss of antigenicity in adsorbed antigens, inhibition of degradation of antigen in cultures was investigated. A number of diverse substances, including ascorbic acid, sodium thioglycolate, glutathione, sodium sulfite, cysteamine, cystamine, citrate, dimercapto propanol (BAL), 8-hydroxyquinoline, ethylenediaminetetraacetate (EDTA), and glyoxal, were added individually, at a concentration of 0.005 M, to culture media. EDTA and BAL inhibited growth when added to the medium prior to inoculation; they were, therefore, added to 48-hr-old cultures, and the cultures were reincubated under nitrogen. The test cultures plus controls were incubated for 96 hr; after this period, a pronounced loss in antigen activity had occurred in control cultures, as shown previously (Puziss and Wright, 1959). Of the compounds tested, EDTA, sodium thio-

TABLE 3. *Inhibition of antigen degradation by additions to anaerobic cultures of Bacillus anthracis*

Addition to culture*	Incubation period	Immunizing activity in guinea pigs†	Complement-fixation titer
	days		*50% units/ml*
Controls (no additions)	2	14/16	80
	4	0/16	20
	5	0/16	8
Ethylenediamine-tetraacetate	4	4/8	80
	5	4/8	60
Sodium thioglycolate	4	3/8	40
Sodium sulfite	3	4/8	40
Glyoxal	5	2/8	40

* All substances were added to a final concentration of 0.005 M. EDTA was added to 48-hr growing cultures, and the cultures were reincubated anaerobically for the remainder of the period shown.

† Immunizing activity is expressed as the number of animals surviving divided by the total number challenged.

TABLE 4. *Effect of various substances on stability of gel-adsorbed antigen at 37 C*

Substances added to adsorbed antigen	Immunizing activity*	
	Initial	After 7 days at 37 C
Control (no additions)	9/12	4/16
Thimerosal (1:10,000)	29/36	3/36
Formalin (0.009%)	23/27	33/39
Thimerosal (1:10,000) + formalin (0.009%)	15/19	9/16
Benzethonium chloride (1:40,000)	4/4	3/4
Benzethonium chloride (1:40,000) + formalin (0.009%)	21/24	20/24
Methyl parabenzoic (1.2 mg/ml) and propyl parabenzoic acids (0.15 mg/ml) + formalin (0.009%)	18/24	19/22

* Antigen preparations for immunization were diluted 1:10 with saline. Immunizing activity is expressed as the number of rabbits surviving divided by the total number challenged.

glycolate, sodium sulfite, and glyoxal were the most efficacious in preventing loss of protective antigen in the culture filtrates (Table 3). Maintenance of an alkaline pH in a culture during prolonged incubation was not, in itself, sufficient to prevent inactivation of antigen.

Accelerated aging was used to estimate the stability of gel-adsorbed antigens, and to test the effectiveness of possible stabilizers. Antigens were incubated at 37 C for 7 days and tested for antigenicity in rabbits at a 1:10 dilution. Addition of 0.005% protamine sulfate to the adsorbed antigen appeared to inhibit the destruction of antigen under these conditions. Further study revealed that preparations of antigen, to which no preservative had been added, deteriorated somewhat less rapidly at 37 C than preparations containing thimerosal (Table 4). Other preservatives acceptable for use in biologicals were investigated. Benzethonium chloride (1:40,000), or a combination of 0.12% methyl parabenzoic acid and 0.015% propyl parabenzoic acid, were tested, and had no adverse effect on the stability of antigen. Benzethonium chloride was selected for use, because the necessary concentration was more readily soluble.

Addition of 0.009% formalin to gel-adsorbed antigen, preserved with benzethonium chloride, or the combination of parabenzoic acids was found to stabilize the antigen during accelerated aging (Table 4). Two preparations of gel-adsorbed antigen, containing formalin and benzethonium chloride, were held at 37 C, and samples were removed at intervals up to 40 days and tested for antigenicity in groups of eight rabbits. The results indicated that after about 25 days the antigens retained sufficient antigenicity to protect 50% of the animals (Table 5). An aerobic, alum-precipitated antigen was found to be of similar stability under the same conditions.

Immunizing activity of stabilized preparation. A comparison of representative lots of gel-adsorbed anaerobic-culture antigen and alum-precipitated aerobic-culture antigen, with respect to their antigenicity in rabbits, is shown in Table 6. Immunization of guinea pigs and mice with the gel-adsorbed antigen was also studied. Guinea pigs were injected subcutaneously with a single 0.25-ml injection of antigen and challenged after 2 weeks with 1,000 spores of the Vollum strain of *B. anthracis*. Mice were immunized by the intraperitoneal route with antigen injected in a volume

TABLE 5. *Stability of preparations of protective antigen at 37 C*

Type of prepn	Days at 37 C	Immunizing activity in rabbits*
Aerobic (lot 2481)	0	4/4
	15	4/4
	33	0/6
Anaerobic (lot 2479-X)	0	6/6
	15	6/6
	28	2/4
	44	0/5
Anaerobic (lot 2456-7)	0	3/4
	15	3/4
	30	2/6

* Samples of antigen were removed after the indicated interval at 37 C and diluted 1:10 with saline for immunization of rabbits. Immunizing activity is expressed as the number of animals surviving divided by the total number challenged.

of 0.5 ml; multiple injections were given 7 days apart. Seven days after the last injection of antigen, the mice were challenged with 100 spores of the Vollum strain, given subcutaneously in a dose of 0.25 ml. The results (Table 7) indicate that both animal species could be readily immunized. More antigen was required than for immunization of rabbits, since this antigen preparation, when diluted 1:10, protected all of four rabbits

Exploratory studies revealed that the gel-adsorbed anaerobic culture antigen may be combined with aluminum phosphate adsorbed pentavalent botulinum toxoid (Cardella, Fiock, and Wright, 1958). Antigenicity of all components was retained at a satisfactory level.

Thorne, Molnar, and Strange (1960) showed that toxin was present in aerobic cultures of *B. anthracis* grown in casein hydrolysate medium. The possibility of factor I of the toxin being present in anaerobic cultures in 1095 medium was investigated. The sintered-glass filters used in

TABLE 6. *Comparative effectiveness of preparations of protective antigen produced under aerobic and anaerobic conditions*

Type of antigen	Range of in vitro titers of culture filtrate		Immunizing activity of adsorbed preparations in rabbits*	
	Complement fixation	Agar diffusion titer	Dilution of antigen injected	Survival ratio†
	50% units/ml			
Aerobic (alum-precipitated)	40–80	1:1–1:4	1:2	24/26
			1:5	17/24
			1:10	14/26
Anaerobic (gel-adsorbed)	160–320	1:4–1:16	1:10	58/67
			1:30	16/27
			1:60	5/12
			1:90	13/28

* Pooled data from a number of preparations of antigen.

† Survival ratios refer to the number of animals surviving divided by the total number challenged.

TABLE 7. *Immunization of guinea pigs and mice with gel-adsorbed protective antigen*

Dilution of antigen	Guinea pigs*			Mice		
	No. of injections	Survival ratio†	Avg day of death	No. of injections	Survival ratio†	Avg day of death
Undiluted	1	6/8	9.0	1	8/9	4.0
1:2	1	7/8	11.0	2	8/10	9.0
1:5	1	4/8	8.2	3	5/10	7.3
1:10	1	1/8	4.4	—	—	—
Unimmunized controls	—	0/8	3.2	—	0/10	3.3

* Immunization and challenge of guinea pigs was by standard methods; for immunization and challenge of mice, see the text.

† Ratios refer to the number of animals surviving divided by the total number challenged.

preparation of the anaerobic culture filtrate were eluted with 0.15 M carbonate buffer at pH 9.6, and the eluates were examined for the presence of toxin. In another experiment, normal horse serum was added to anaerobic cultures, prior to filtration, to allow passage of toxin through the filter. The filtrates were examined for the presence of toxin by the guinea pig skin test and the agar gel diffusion method (Thorne et al., 1960). No factor I could be demonstrated either in the eluates from the glass filters or in the serum-containing filtrates.

Trial in man. To study the response to the antigen in man, two groups of volunteers were immunized, one with the alum-precipitated aerobic antigen, and the other with the gel-adsorbed anaerobic antigen. Three 0.5-ml injections of antigen were given subcutaneously in the deltoid region on a 0-, 2-, and 4-week schedule. The rates of occurrence of erythema, edema, or pruritis at the site of injection were low in both groups, and did not appear to be different. No systemic reactions were observed. Serological study of the responses to the two antigens is in progress.

Discussion

The present data explain neither the failure of alum to precipitate the protective antigen in filtrates of anaerobic cultures, nor the highly satisfactory results obtained with preformed aluminum hydroxide gel. The use of aluminum hydroxide for adsorption of antigen was described by Hektoen and Welker (1933), and the gel has since been used as an adsorbent and adjuvant in preparations for immunization of man (U.S. Dispensatory, 25th ed., 1955). Aluminum hydroxide, produced according to the method of Hansen (1953), was adopted for routine use, because it was prepared according to published methods, specifically for use in human biologicals.

Stability in storage at 4 C of the initial preparations of gel-adsorbed protective antigen was poor. Definite indications were obtained that the presence of thimerosal as a preservative contributed to this instability. This reaction was suggestive of the finding of Davisson et al. (1956) that copper ions present in poliomyelitis vaccine catalyzed the oxidation of thimerosal to substances that destroy the antigenicity of the vaccine. Addition of EDTA to chelate the copper ions prevented the oxidation and consequent inactivation of the vaccine. Addition of EDTA to the gel-adsorbed protective antigen was impractical, however, because of the high concentration of aluminum present. The replacement of thimerosal with benzethonium chloride significantly increased the stability of the adsorbed preparation. Benzethonium chloride has been found acceptable as a preservative in poliomyelitis vaccine (McLean, 1957).

A further increase in stability of the adsorbed preparation was desirable, and various substances were tested for stabilizing activity. Previous studies had shown that protective antigen disappeared rapidly from whole cultures when they were incubated beyond the optimal time. Strange and Thorne (1958) showed that a peptidase and a gelatinase may be involved in the degradation. The present study indicated that addition of EDTA after growth of the culture inhibited degradation of antigen during prolonged incubation, presumably by chelating metallic ions essential for enzyme action. The other compounds that prevented degradation of antigen in cultures presumably also acted by inhibition of enzyme action. None of these substances, however, was desirable in a parenteral product.

Protamine sulfate has been used as a stabilizer and adjuvant of parenteral insulin (Hagedorn et al., 1936), and as a binding agent in combinations of toxoid and aluminum phosphate gel (Holt, *personal communication*). Although small amounts of protamine sulfate stabilized the anaerobic gel-adsorbed antigen, protamine is not a well-characterized substance of constant composition, and precedents for its use in human biologicals are not extensive. Satisfactory stability was achieved by the addition of a low concentration of formalin. The gel-adsorbed preparation containing benzethonium chloride and formalin appeared to be somewhat more stable than the alum-precipitated aerobic culture product at 37 C. Since the latter showed satisfactory stability at 4 C, it is probable that the former will also prove satisfactory under these conditions. The gel-adsorbed deep-culture product showed significantly greater immunizing ability than the aerobic alum-precipitated product in animals, and was tolerated in man at least as well as the aerobic antigen.

The absence of factor I of the anthrax toxin in filtrates of anaerobic cultures is significant with respect to the safety of the anaerobic antigen for parenteral use in man. The anaerobic cultures

gave no evidence of the presence of factor I, either on gel diffusion plates or by the guinea pig skin test. Thorne et al. (1960) and Sargeant, Stanley, and Smith (1960) demonstrated the presence of toxin in other cultures of *B. anthracis*; the reason for the absence of factor I in the present cultures is unknown. It may be that use of the chemically defined medium, the anaerobic culture conditions, or the low pH developed during growth inhibited accumulation of factor I. Factor III, the lethal factor of Stanley and Smith (1961) and Beall, Taylor, and Thorne (1962), had not been reported at the time this work was completed.

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